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Localization of the complement regulatory proteins in the normal human kidney

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Localization of the complement regulatory proteins in the normal human kidney. The kidney is an organ where complement-mediated tissue injuries take place by various stimuli. To assess how the kidney is protected from the autologous complement attack, comparative localization of decay accelerating factor (DAF), membrane cofactor protein (MCP) and 20 kDa homologous restriction factor (HRF20) was studied in the normal human kidney. Specific monoclonal antibodies to DAF, MCP and HRF20 were used for the study. Studies by immunofluorescence and immunoelectron microscopy showed that the distribution of each protein in the kidney was complementary to each other in most parts. MCP and HRF20 were clearly seen in the glomerular capillaries, while DAF was only faintly observed. Juxtaglomerular apparatus was abundant in DAF and MCP but not in HRF20. HRF20 was most strongly expressed in the peritubular capillaries where MCP was not detectable. Basolateral membranes of the proximal tubules and collecting ducts expressed MCP strongly, while there was no expression of DAF in the proximal tubules. Interestingly, both DAF and MCP, which inhibit complement activation at C3/C4 level, were not expressed in the apical portion of the tubular cells including proximal tubule brush border. In contrast, HRF20 was expressed on the apical part of the tubules. Medullary interstitium strongly expressed MCP but not DAF. Based on these observations, we conclude that each segment of the kidney is protected from the complement attack by the different combination of complement regulatory proteins. We speculate that the tubular cells might be fragile when complements are activated inside the tubular lumen, because there is no expression of complement regulatory proteins which inhibit C3 convertase.

The complement system plays an important role in self defense. Its cascade is activated not only by antigen-antibody interaction but also by non-immunological stimuli [1], resulting in release of anaphylatoxins [2] or the formation of membrane attack complex (MAC) on the target cell membrane. The intact host cells, however, are protected from indiscriminate attack of the autologous complement system. This is mediated in part by a relatively non-specific self-recognition system of the alternative pathway [3] and in part by specific complement regulatory proteins present on the cell membranes [4].

Mechanisms by which the kidney is injured by the complement system have been studied extensively. It is now clear that the kidney is always under the menace of complement attack. The glomerulus has a function and structure by which macromolecules, including immune complexes, can be easily trapped and thus activate the complement system. It has been shown in experimental glomerulonephritis that complement depletion by cobra venom factor ameliorated immune-mediated glomerular injuries [5]. Tubules are directly exposed to various complement components in proteinuric condition. Since the brush border of proximal tubules can activate alternate pathways of complement both in tissue section [6] and in cells in culture [7], the presence of complement components in the tubular lumen may lead to complement attack on the tubular cells [8]. In addition, ammonium concentration is markedly high in renal medulla [9]. Since ammonium can activate C3 by disrupting the internal thioester bond [10, 11], there is possibility that the alternate pathway is spontaneously activated in the renal medulla. Ammonium production is markedly increased after protein diet [12] and is inhibited by the administration of sodium bicarbonate [13]. In an experimental model, inhibition of ammonium production by sodium bicarbonate protected the kidney from progressive renal failure [13]. Thus, ammonium seems to play an important role in the progression of renal disease through mediation of complement activation. The acidic condition seen in renal medulla may also activate C3. All of these events might cause serious renal injury if there is no inhibitory mechanism against complement activation.

Recently, a family of complement regulatory proteins present on the cell membranes has been identified in the human, and they are widely distributed not only on the membranes of blood cells but also in the vascular endothelum and extra-vascular tissues [14–17]. These include decay accelerating factor (DAF or CD55) [18], membrane cofactor protein (MCP or CD46) [19] and 20 kDa homologous restriction factor (HRF20 or CD59) [20, 21]. DAF and MCP inhibit the complement activation cascade at C3 convertase level, and HRF20 inhibits the formation of membrane attack complexes (MAC). Although localization of these molecules in the kidney has been reported [14–16, 22–25], the precise comparative localization has not been studied yet. In this study, we investigated comparative localization of DAF, MCP and HRF20 in normal human kidneys in an attempt to evaluate the role of each molecule in the defense against autologous complement attack.

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Methods

Kidney specimens

Specimens from normal human kidneys were studied. They were prepared from the normal portion of nephrectomized kidneys of four patients who had renal tumors. These specimens were confirmed as histologically normal by light microscopic examination.

Antibodies

Mouse monoclonal antibodies against human DAF (IA10) and MCP (M75) were gifts from T. Kinoshita (Department of Immunochemistry, Research Institute for Microbial Deseases, Osaka University School of Medicine, Suita, Japan) and T. Seya (Department of Immunology, The Center for Adult Deseases, Osaka, Japan), respectively. A monoclonal antibody against human HRF20 (1F5) was prepared by Okada and co-workers. These monoclonal antibodies inhibit the function of corresponding regulatory molecules. Specificity and property of IA10, M75 and 1F5 have been previously reported [20, 26, 27]. IA10 recognizes a 70 kDa human DAF molecule by Western blot analysis. IA10, when used in combination with IIH6 (another anti-DAF monoclonal antibody), inhibited the function of DAF by 78% [26]. M75 immunoprecipitates human MCP, and it blocks MCP factor I-cofactor activity by 70% [27]. 1F5 recognizes HRF20 and it blocks the function of HRF20 in a dose-dependent manner [20]. All of these antibodies belonged to mouse IgG1 subclass.

To identify the precise segments of tubules and collecting ducts expressing these three antigens, double immunofluorescence staining method was employed using the following antibodies. One is goat antibodies against human Tamm Horsfall protein (THP or uromucoid) (SEROTEC Ltd., Oxford, UK). THP is present in the distal tubules of cortex and outer medulla [28, 29]. Others are serum obtained from a patient with anti-tubular basement membrane (anti-TBM)-mediated tubulointerstitial nephritis [30, 31], and a monoclonal antibody (H79B12) reactive with the nephritogenic 54 kDa antigen of bovine TBM [31]. Both predominantly bound to the proximal TBM of the normal human kidney by indirect immunofluorescence.

Immunofluorescence studies

Small pieces of kidney cortex and medulla were embedded in OCT compound (Miles Inc., Elkhart, Indiana, USA) and were snap-frozen in liquid nitrogen. Frozen tissues were cut by a cryostat and 2-μm thick sections were obtained. They were fixed in acetone for 15 minutes at room temperature. Studies were performed by an indirect immunofluorescence (IF) technique [32]. For the staining of DAF, MCP, HRF20, and proximal TBM, the sections were washed with PBS and then incubated at room temperature for 15 minutes with one of mouse monoclonal antibodies against complement regulatory proteins. As a control, the sections were incubated with an irrelevant monoclonal antibody of IgG1 subclass, H38F9E6A6. The optimal concentrations of IA10, M75, 1F5 and H79B12 were 10 μg/ml, 2.5 μg/ml, 12 μg/ml and 10 μg/ml, respectively. The sections were washed three times with PBS and they were then incubated with fluorescein isothiocyanate (FITC)-labeled rabbit anti-mouse IgG antibodies (Zymed Laboratories, San Francisco, California, USA) absorbed with normal human serum. For the double immunofluorescence staining of THP and complement regulatory protein, the sections were first stained for one of the complement regulatory proteins as described above. They were then incubated with goat anti-human THP antisera (diluted at 1:250) followed by the incubation with rhodamine-labeled rabbit anti-goat IgG (Cappel, Westchester Pennsylvania, USA) absorbed with normal human

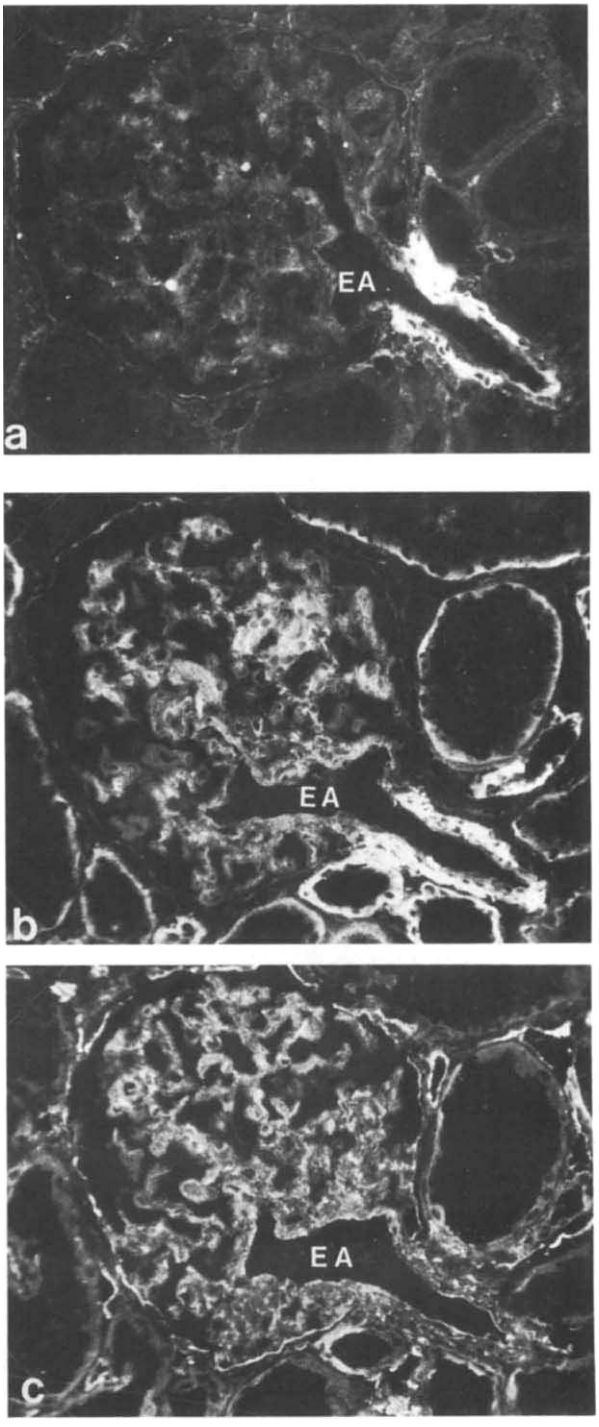


Fig. 1. *Immunofluorescence micrographs showing localization of DAF* (**a**), *MCP* (**b**) *and HRF20* (**c**) *in the glomerulus and efferent arteriole (EA).* (a, b, c: ×200)

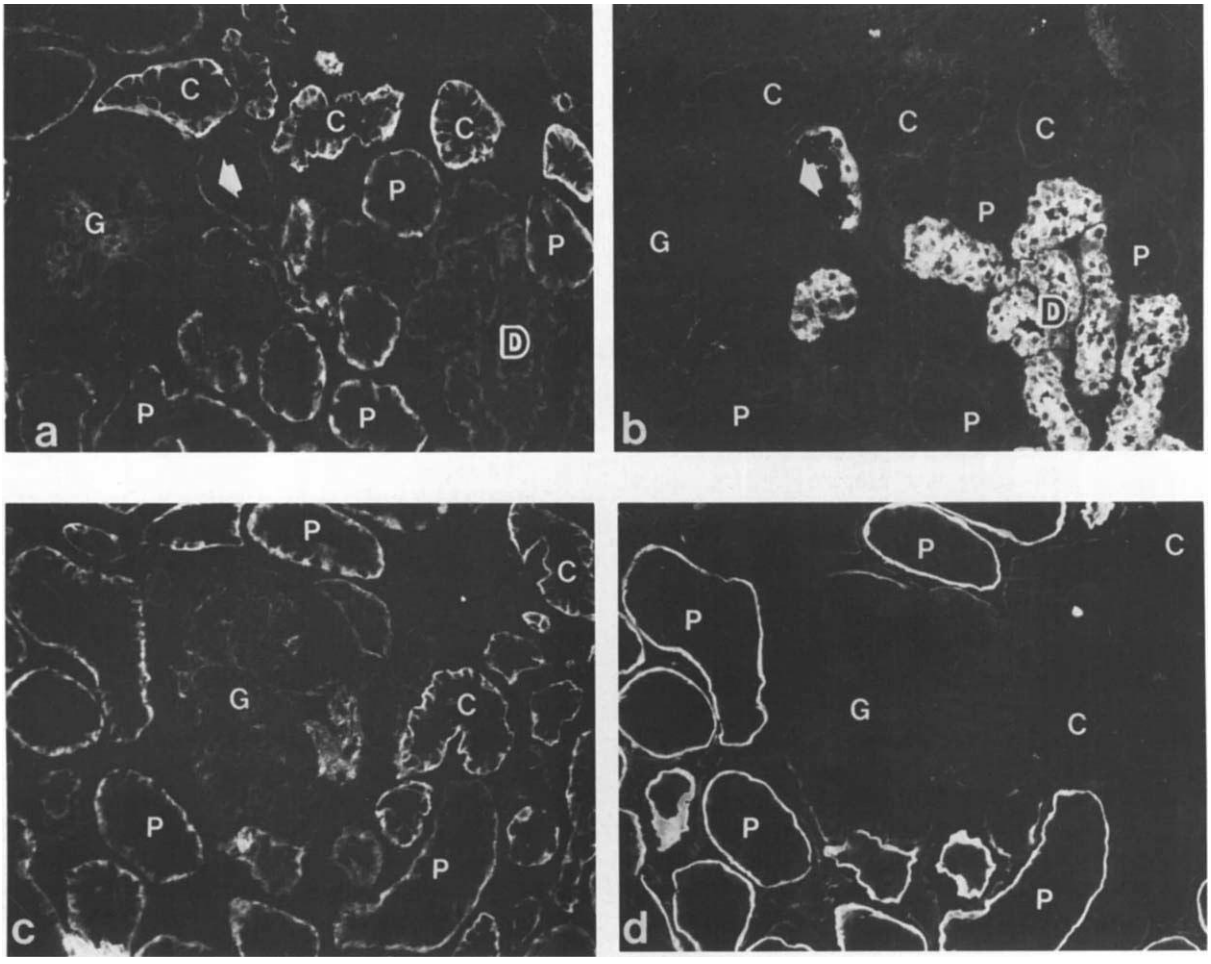


Fig. 2. *Double immunofluorescence micrographs.* Two pairs of figures (a vs. b, and c vs. d) indicate the same tissue sections. (**a** and **c**): The localization of MCP in the cortex. (**b** and **d**): The localization of THP and proximal TBM, respectively. Arrows indicate macula densa. These pictures show an example how each nephron segment is identified. The segment which are most strongly stained for MCP is not reactive with anti-THP and anti-proximal TBM antibodies. Thus the segments most strongly stained for MCP are identified as collecting ducts. Segments reactive with anti-THP or with anti-proximal TBM are identified as distal tubules or proximal tubules, respectively. Abbreviations are: G, glomerulus; P, proximal tubule; D, distal tubule; C, collecting duct. (a, b, c, d: ×100)

serum. For the double immunofluorescence staining for TBM and complement regulatory proteins, frozen sections were first treated with 0.1% streptoavidin (Gibco BRL, Gaithersburg, Maryland, USA) for 10 minutes and 0.01% biotin (Vector Laboratories, Burlingame, California, USA) for 10 minutes at room temperature. They were then incubated with an antibody against one of the complement regulatory proteins followed by the incubation with rhodamine-labeled rabbit anti-mouse IgG (Cappel). After washing with PBS, sections were treated with normal mouse serum (1:100 dilution). They were then incubated with biotinylated H79B12 or rhodamine-labeled human anti-TBM antibodies followed by the incubation with FITC-labeled streptoavidin (Vector Laboratories). After the final wash with PBS, all the sections were mounted with medium containing *p*-phenylenediamine [33] and examined by an Olympus BH-2 epifluorescence microscope. Since peritubular capillaries were most strongly stained by 1F5, we assigned the staining intensity of the peritubular capillaries as 3+ and the intensity of immunofluorescence staining was graded from 0 (negative) to 3+ accordingly. Similarly, the intensity of immunofluorescence staining for DAF of juxtaglomerular apparatus and that of MCP of cortical collecting tubules and smooth muscle cells of afferent arterioles were assigned 3+. The staining intensity for DAF and MCP of the other portion of the kidney was graded from 0 (negative) to 3+ accordingly.

Immunoelectron microscopy (IEM) studies

To see the localization of MCP and HRF20 in the kidney cortex, IEM studies were carried out according to a method previously described [32]. IEM studies were not performed for DAF because immunoreactivity of tissue DAF with IA10 was lost by various fixation methods [22]. Small blocks of normal human kidney were fixed with periodate lysine paraformaldehyde (PLP) fixative [34] for four hours at 4°C, washed with 10 mM PBS containing 10%, 15% and 20% sucrose. After final wash in PBS containing 20% sucrose and 5% glycerol for one hour, blocks were embedded in OCT compound and frozen in liquid nitrogen. Six-micrometer thick frozen sections were mounted on albumin-coated slides and treated with PBS containing $NaBH_4$ (50 mg/dl) for 40 minutes at room temperature.

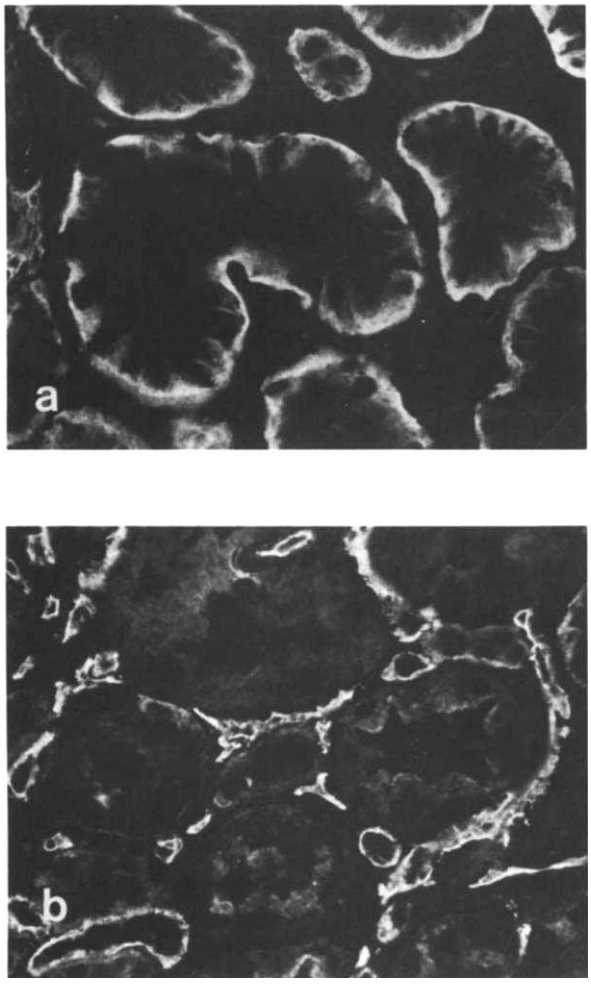


Fig. 3. *Immunofluorescence micrographs showing localization of MCP (a) and HRF20 (b) in the proximal tubules and peritubular capillaries.* (a, b: ×400)

After washing with PBS containing 10% sucrose, sections were incubated with normal horse serum for 30 minutes at room temperature, and then incubated with the first antibody (M75 or IF5) overnight at 4°C. For the detection of mouse IgG sections were first incubated with biotinylated horse anti-mouse IgG and then with peroxidase labeled streptoavidin (Vectastain ABC Kit, Vector Laboratories). The sections were post-fixed with 2.5% glutaraldehyde and incubated in 0.5 M Tris-HCl buffer containing 0.02% diaminobenzidine tetrahydrochloride for 30 minutes at room temperature. They were incubated for another 1 to 2 minutes in 0.05% H_2O_2 and were further fixed with OsO_4. They were then flat embedded in Epon 812. Ultra thin sections were stained with lead for 15 seconds and were observed by a JEOL-100 CX electron microscopy (JEOL Company Ltd., Tokyo, Japan).

PIPLC treatment

To analyze how DAF, MCP and HRF20 are anchored to the cell membrane of the kidney, we treated the sections with 4 mg/ml phosphatidylinositol specific phospholipase C (PIPLC, Toa Gousei Chemical Industry Co. Ltd., Tokyo, Japan) for 60 minutes at 37°C before incubating the sections with first antibodies [35, 36].

Results

Cortex

Glomerulus. HRF20 was moderately expressed in glomerular capillaries and in Bowman's capsule, and it was weakly expressed in the juxtaglomerular apparatus. In contrast, DAF and MCP were moderately to strongly expressed in juxstaglomerular apparatus (not shown). DAF and MCP were weakly expressed in glomerular capillaries and Bowman's capsule (Fig. 1).

Tubule. In the cortex proximal tubules were identified by H78B12 and distal tubules by anti-THP serum. Cortical collecting ducts or connecting tubules were identified as segments which did not react with these two antibodies (Fig. 2). There was no expression of DAF in the proximal and distal tubules. Basal membrane of the cortical collecting ducts only weakly expressed DAF (figure not shown). MCP was most strongly expressed in basolateral membranes of cortical collecting ducts, and moderately in the same area of proximal tubules (Fig. 3a). In contrast to these areas, apical portion (brush border) of proximal tubules did not express MCP (Figs. 3a and 4 a, b). Distal tubules weakly expressed MCP. HRF20 was most strongly expressed in the peritubular capillaries (Fig. 3b), while MCP was absent in this area (Fig. 3a). HRF20 was weakly expressed in the brush border of proximal tubules (Figs. 3b and 4 c, d).

Vessels. Smooth muscle cells of arterioles strongly expressed DAF and MCP, while expression of HRF20 was much weaker in the same area. Endothelium of arterioles, however, expressed HRF20 moderately but not DAF or MCP (Fig. 1).

Outer medulla

Outer stripe. Localization of DAF, MCP and HRF20 in each segment of the kidney was similar to that observed in the cortex.

Inner stripe. Vascular bundles are fully developed in this area. Although vascular walls of descending vasa recta were strongly stained for DAF, MCP and HRF20, localization of HRF20 was more confined to the endothelial aspects of vascular walls. In contrast, ascending vasa recta expressed only HRF20 but not DAF or MCP (Fig. 5). Nephron segments present in the inner stripe of the kidney include distal tubules (straight part), collecting ducts and thin limbs of Henle. DAF was observed in the basal membranes of collecting tubules but not in distal tubules or thin limbs of Henle. DAF was moderately expressed in peritubular capillaries (not shown). MCP was strongly expressed in the collecting tubules, but it was only faintly expressed in the distal tubules, thin limbs or peritubular capillaries. MCP was also present in the interstitium (not shown). HRF20 was strongly expressed in the peritubular capillaries. It was weakly expressed in the apical part of distal tubules and in the surface of collecting ducts (not shown).

Inner medulla

DAF was present in the vessels and the basal membrane of collecting ducts. It was not observed in most part of interstitium (lipid laden interstitial cells or type 1 medullary interstitial cells;

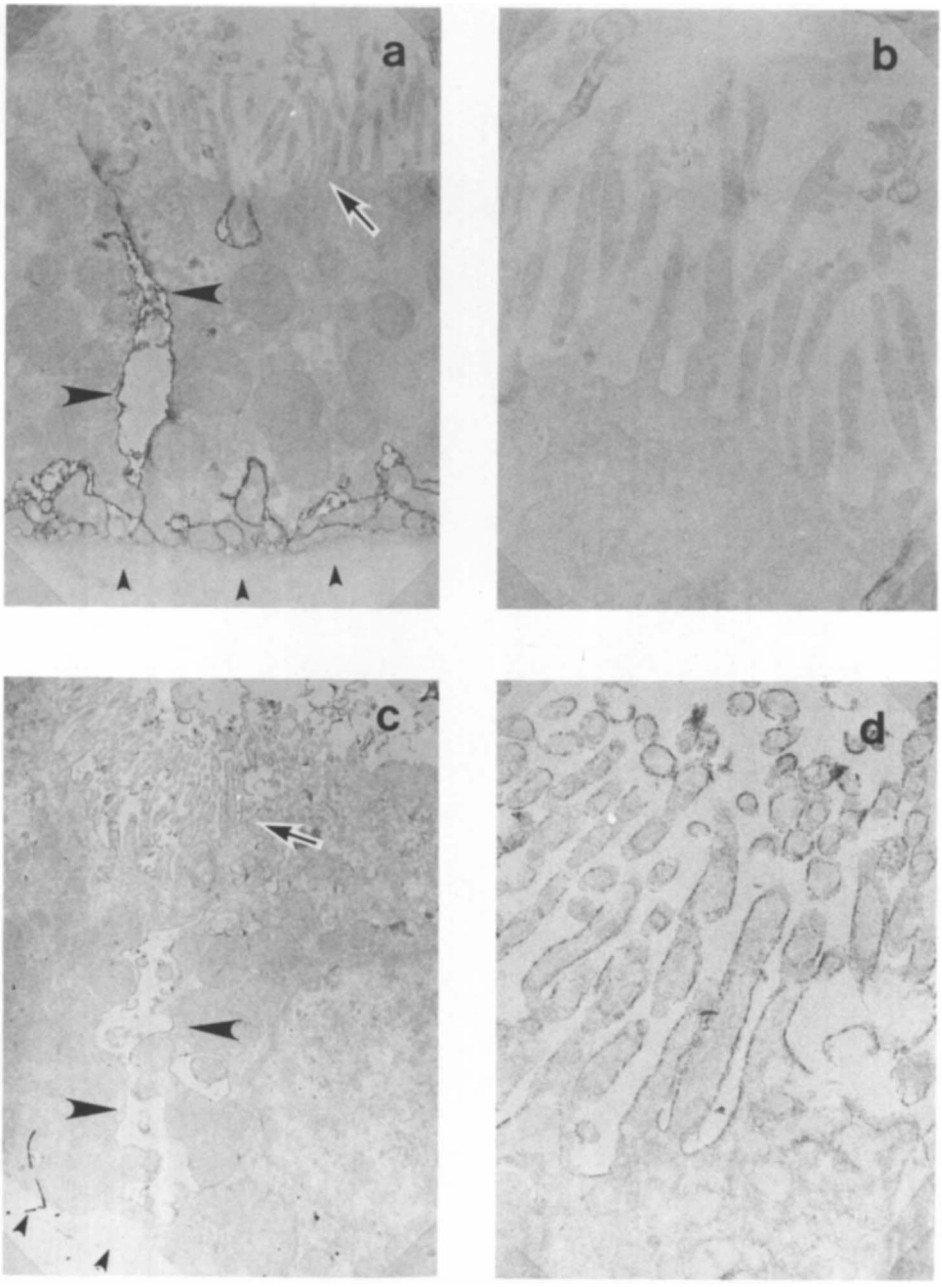


Fig. 4. *Immunoelectron microscopy showing localization of MCP (***a, b***) and HRF20 (***c, d***) in the proximal tubules.* Note that MCP is moderately expressed in the basolateral membrane of the proximal tubules while it is not present in the brush border. HRF20 is weakly expressed in the tubular brush border. Symbols are: arrows, brush border; small arrowheads, TBM; large arrowheads, lateral membrane. (a: ×2,600, b: ×5,000, c: ×2,000, d: ×6,300)

Fig. 6a). MCP was strongly expressed in the interstitium as well as in the baso-lateral membrane of collecting tubules and in the vessels (Fig. 6b). HRF20 was again strongly expressed in the capillaries. It was weakly expressed in the interstitium and in the collecting ducts (Fig. 6c).

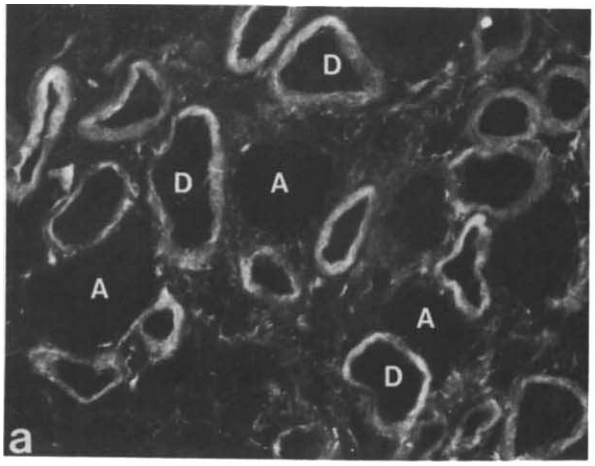
These data are summarized in Table 1.

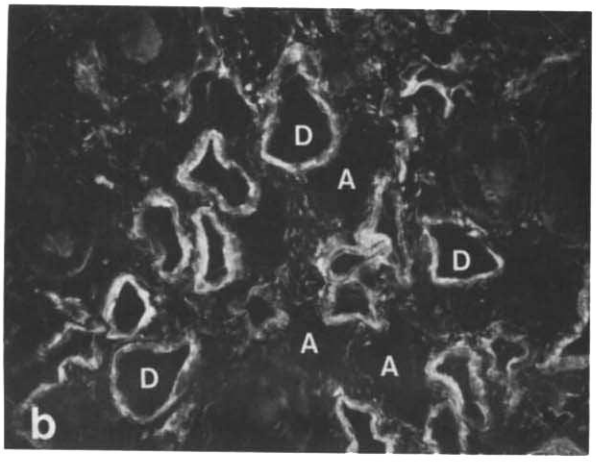
PIPLC treatment

By PIPLC treatment, the staining for HRF20 became barely detectable. The influence of PIPLC treatment on the staining for DAF was also observed although the effect was relatively less prominent. By way of contrast, staining for MCP was not affected at all by PIPLC treatment.

Discussion

In the present work we studied comparative localization of DAF, MCP and HRF20 in the normal human kidney in order to assess how the kidney is protected from autologous complement attack. These molecules are known to be widely distributed throughout the body, while other regulatory proteins such as complement receptor 1 and 2 (CR1 and CR2) are basically limited to blood borne cells [37]. Thus it is thought that DAF,





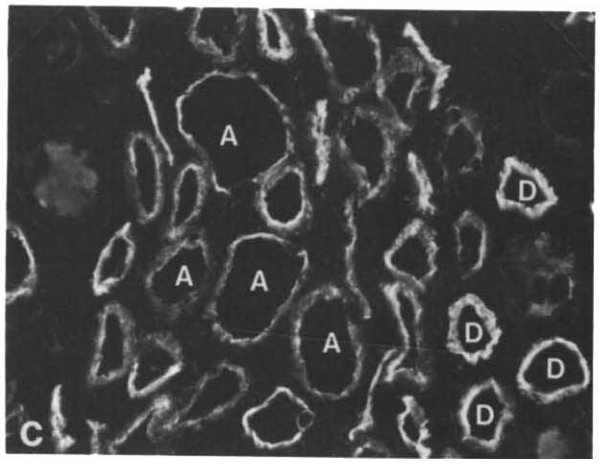
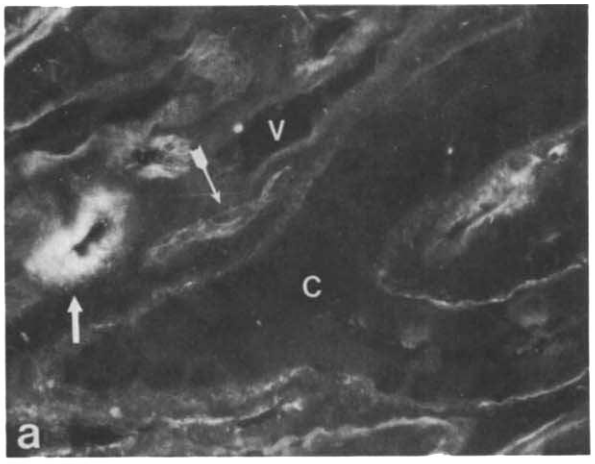
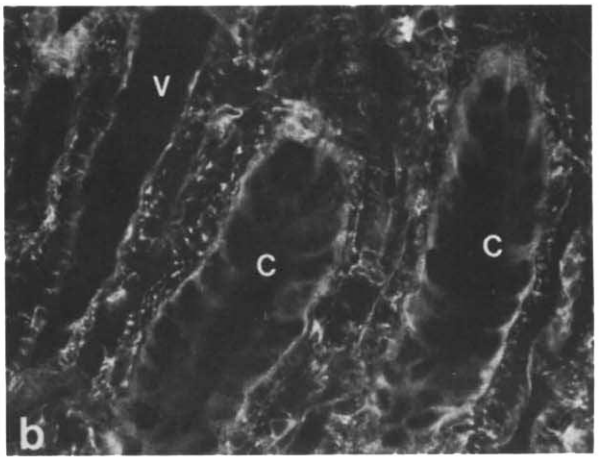


Fig. 5. *Immunofluorescence micrographs showing localization of DAF* (**a**), *MCP* (**b**), *and HRF20* (**c**) *in the area of vascular bundles of the inner stripe*. Abbreviations are: D, discending vasa recta; A, ascending vasa recta. (a, b, c: ×400)





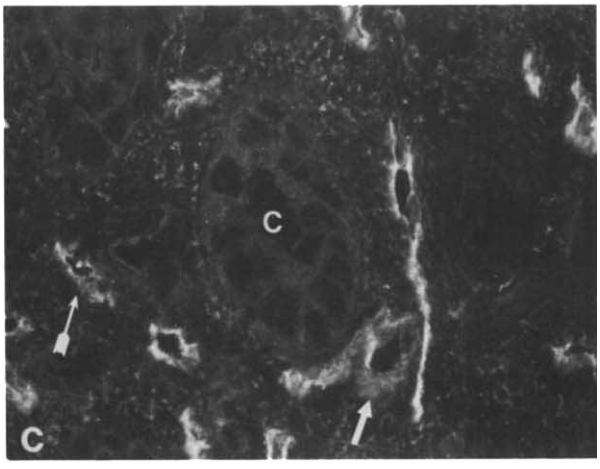


Fig. 6. *Immunofluorescence micrographs showing localization of DAF* (**a**), *MCP* (**b**) *and HRF20* (**c**) *in the inner medulla*. Abbreviations and symbols are: C, collecting duct; V, vessels; thin arrows, peritubular capillaries; thick arrows, arterioles. (a, b, c: ×400)

MCP and HRF20 play major roles in the protection against complement attack on the kidney cells.

From the present work we obtained three new findings. First, DAF, MCP and HRF20 have different localizations in the normal human kidney. Although there was limitation in the quantitation of each molecule, apparently there was difference in the staining intensity in the different segment of the nephron. HRF20 was most widely distributed in the kidney. MCP and DAF seemed to compensate with each other. From the viewpoint of defense against complement attack, this observation is

Table 1. Summary of localization of complement regulatory proteins in the normal human kidney

Segment	DAF (CD55)	MCP (CD46)	HRF20 (CD59)
Glomerular capillaries	+/−	+ ~ ++	++
Bowman's capsule	+/−	+	+ ~ ++
Juxtaglomerular apparatus	+++	++	+
Proximal tubules			
Basolateral membrane	−	++	+/−
Bush border	−	−	+
Distal tubules			
Basolateral membrane	+/−	+	+
Apical membrane	−	−	+
Collecting ducts ~ Connecting tubules			
Basolateral membrane	+ (basal membrane)	+ ~ +++ (inner medulla ~ cortex)	+/−
Apical membrane	−	−	+/−
Intermediate tubules (Thin limb of Henle)	−	+/−	+/−
Peritubular capillaries	+	−	+++
Arterioles			
Smooth muscle cells	++	+++	+
Endothelium	+/−	+/−	++ ~ +++
Vascular bundles			
Descending vasa recta	++	++ ~ +++	+++
Ascending vasa recta	−	−	+ ~ ++
Medullary interstitium (Interstitial cells)	−	++	+

reasonable because each segment regulates C3 convertase activity either by DAF or MCP, and inhibits formation of MAC by HRF20. Since these two steps are most important in the regulation of alternative pathway of complement and the subsequent cellular damage, most structures of the normal kidney are protected at these two steps. Second, apical portion of the proximal tubular cells were free of DAF and MCP, both regulators of C3 convertase. Our findings concerning DAF localization are consistent with those of Cosio and coworkers using biopsy specimens [22]. Although Medof showed positive staining for DAF in the tubular epithelial cells of autopsy kidneys [14], expression of DAF in the tubular brush border was not well described. Our observation is interesting because tubular brush border is reported to activate the alternate pathway of complement [6]. It is also reported that proteinuria causes tubular damage in rats [38]; there is evidence of complement activation in the proximal tubular epithelium in patients with nonselective proteinuria [39]. One of the reasons why complements are activated in the proximal tubule brush border might be related to the absence of both DAF and MCP in this portion. Once C3 is activated by any stimuli, there will be further activation of complements through the amplification system of C3 convertase because there is no inhibitory protein in the proximal tubule brush border. Third, MCP and HRF20 were intensely localized in the interstitium between collecting ducts and vessels in the inner medulla. Since the predominating cells in this area are lipid laden interstitial cells (or type 1 medullary interstitial cells), it was speculated that these cells expressed MCP and HRF20. Although the physiological role of lipid laden interstitial cell is unknown, these cells are also protected from complement attack by the presence of complement regulatory proteins.

Finally the present study indicates that, like in blood cells, HRF20 and DAF are present on the surface of the cell membrane by phosphatidylinositol anchoring, but MCP is not anchored by glycophosphatidylinositol in the normal human kidney. The difference of sensitivity of HRF20 and DAF to PIPLC treatment is thought to be dependent on the cells on which each molecule is expressed [20, 40, 41]. It is further suggested that the different sensitivity of glycolipid-anchored acetylcholinesterases results from presence or absence of acylation of a inositol ring in the glycolipid anchor [42]. The exact nature of the glycophosphatidylinositol anchoring of DAF and HRF20 in the kidney cells should be further investigated.

In conclusion, the present work demonstrates that the normal human kidney is abundant in the membrane-associated complement regulatory proteins, and that most segments of the normal human kidney are protected from autologous complement attack by different combination of three types of complement regulatory proteins.

Acknowledgments

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